METHODS

Systematic Protocol for the Accumulation of Fatty Acid Data from Multiple Tissue Samples: Tissue Handling, Lipid Extraction and Class Separation, and Capillary Gas Chromatographic Analysis

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ABSTRACT

A systematic procedure was developed for detailed fatty acid profiling of both neutral and polar lipid fractions isolated from hundreds of related bovine muscle and adipose tissue samples. A regimen was established for a nonbiased handling of tissue samples, which included their handling in a predetermined random order. Lipid class separation was accomplished concomitantly during the extraction of the tissues by a selective dry column method, which allowed a detailed analysis of minor but important polyunsaturated fatty acids associated with the polar fraction. Neutral lipids were derivatized to fatty acid methyl esters (FAME) by a literature procedure. However, to protect against lysis of plasmalogens in the polar fraction, a modified nonacidic esterification procedure was developed. FAME profiles were obtained on a programmable high resolution capillary gas chromatograph (GC). Run programs for unattended GC operation and data storage are described. By this overall procedure, the quantitation and peak identification were obtained for major and minor fatty acid constituents from bovine tissue in a manner that prepares for valid statistical interpretation of the resulting data.

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This report describes a systematic approach used to gather fatty acid compositional data from a large set of tissue samples. The approach was developed to allow for an unbiased handling of the samples, proper record-keeping, and—most importantly—the timely completion of an otherwise unmanageable task.

This systematic approach was developed and used for 2 bovine dietary studies in conjunction with Oklahoma State University. One study investigated differences in beef quality of animals raised on forage (wheat pasture) compared to those on grain. The other determined the effect of the antibiotic supplement monensin on beef quality (1).

Three goals were considered during the development of the approach. First, fatty acid data needed to be accumulated in such a way as to allow later valid statistical analysis. Tissues were to be comminuted and extracted in random order. Data obtained by gas chromatography (GC) analysis were to be electronically stored for later retrieval and manipulation. Second, data needed to be detailed and accurate. This was to be achieved by the use of complete extraction and proper derivatization techniques, and by efficient GC separations and careful peak identification. Third, data needed to be accumulated in a timely way, despite the large number of tissue samples to be handled. This was to be accomplished by rapid extraction and derivatization techniques and the use of automated GC instrumentation. All the above mentioned criteria were met by the procedures described in this report.

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EXPERIMENTAL

Tissue Samples

Samples of bovine tissue were obtained from Oklahoma State University and consisted of the following: kidney knob fat (KD), *M. longissimus dorsi* (LD), *M. psoas* major (PM), *M. semi tendinosus* (ST), and subcutaneous fat (SQ), all of which were attained from the carcasses of animals used in 2 specific feeding studies. The first of these studies (Fig. 1) required 140 tissue samples and the second 116 samples.

Tissue Handling

Tissue samples were sealed in plastic pouches at Oklahoma State University, shipped to this laboratory in dry ice, and stored at -60 C until analyzed. Samples of ca. 100 g each were comminuted at 10 C in a food processor (Cuisinart CFP-5A, Cuisinarts, Inc., Stamford, CT) together with 30 μl of antioxidant (50% 1:1 BHA/BHT in ethanol, Tennox 5, Eastman Kodak Corp., Rochester, NY). Replicate samples were weighed (5 g $\pm$ 0.1 mg), sealed, and stored at -60 C. To eliminate experimental bias over the several weeks necessary for extraction of all the tissue samples, each sample was coded randomly before lipid extraction was undertaken. Frozen comminuted samples were then removed from storage and extracted on the basis of this sequence.

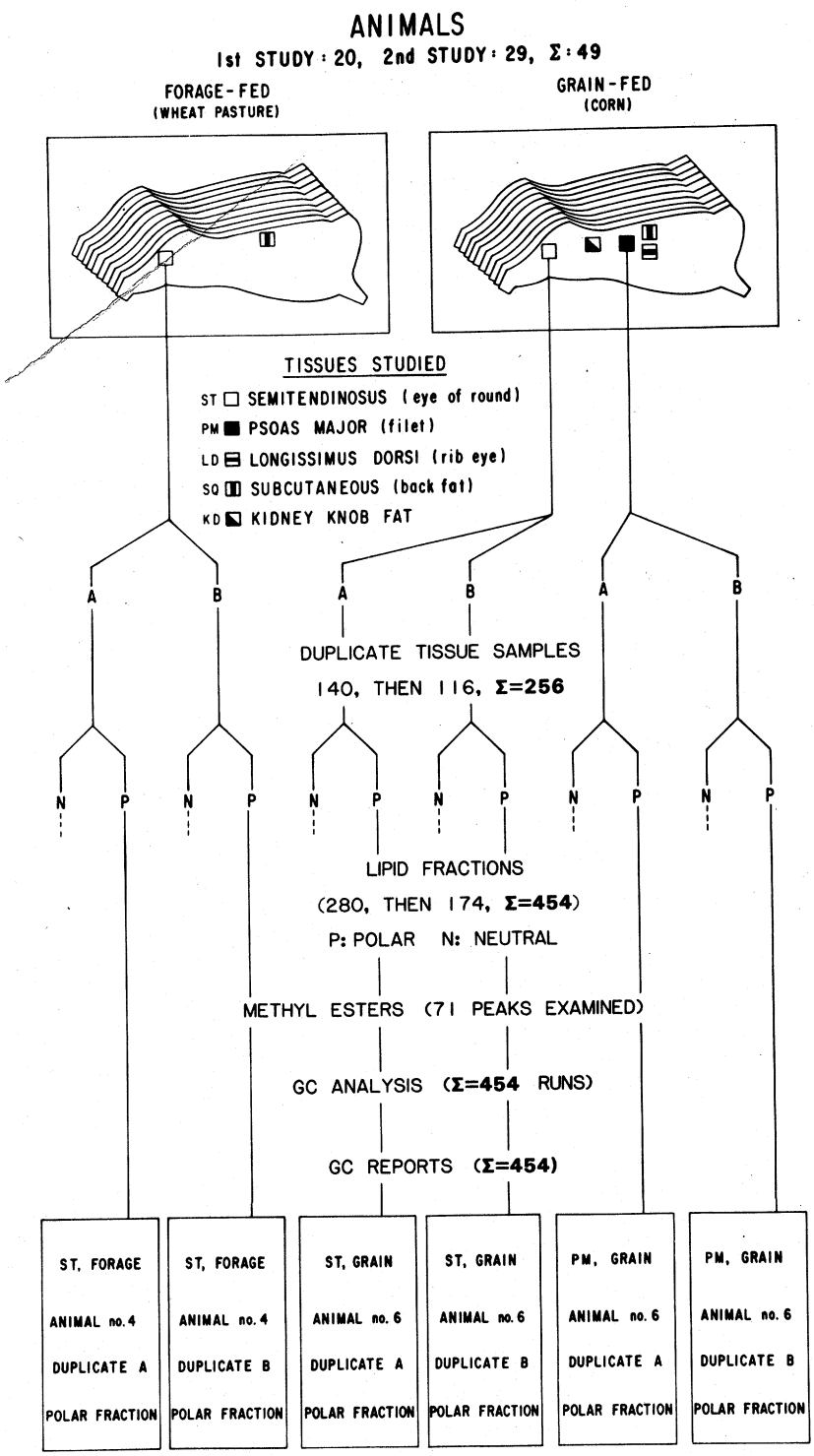

FIG. 1. Flow scheme for the analysis of multiple tissue samples.

Extraction of Lipids

Duplicates of each tissue sample (5 g ± 0.1 mg) were extracted sequentially by the dry column method (2). Before the sample was ground in the mortar, Tennox 5 (1 ml, 0.01 mg in methylene chloride) was added to the tissue. Eluates from each neutral and polar fraction were collected in 200 ml round bottom flasks. Solvent was removed on a rotary evaporator at room temperature, 1 ml of Tennox 5 added, and the contents transferred with hexane to a 100 ml volumetric flask and brought to volume with hexane. Aliquots were taken for thin layer chromatography (TLC), phosphorus analyses, and weight determination. A larger aliquot was reserved for derivatization to FAME's for subsequent GC analysis.

Derivatization of Glycerides to Methyl Esters: Neutral Lipid Fraction

The lipids were converted to their methyl esters by treatment with NaOH/methanol followed by BF_3/methanol (3).

Derivatization: Polar Lipid Fraction

A previously reported procedure (4) was modified in the following manner: an aliquot of the polar lipid fraction containing ca. 20 mg of lipid was first reduced in volume to about 5 ml on a rotary evaporator at room temperature. The contents of the flask were quantitatively transferred to a 15 ml centrifuge tube with a few ml of hexane, and the remaining solvent was removed under a stream of nitrogen. The residue was immediately dissolved in 1.0 ml of isooctane containing 4 mg of the internal standard methyl heneicosanoate followed by addition of 100 μl of 2 N KOH in MeOH (1.1 g/10 ml). The contents of the tube were mixed on a vortex mixer for 60 sec and then centrifuged to separate the layers. The lower methanol layer in the tube was removed with a microsyringe and discarded. A saturated ammonium acetate solution (0.5 ml) was added to the tube, the tube's contents stirred on the vortex mixer, centrifuged, and the aqueous layer removed and discarded. The above procedure was repeated with 0.5 ml of water instead of ammonium acetate. A small amount of anhydrous sodium sulfate was placed in the tube, the contents were stirred and allowed to stand for 30 min, and then the mixture was centrifuged. The upper isooctane layer (ca. 1 ml) of FAME was transferred to a 1 ml serum bottle (No. 223682, Wheaton Glass Co., Millville, NJ), which was then sealed with a cap lined with Viton A (Hewlett-Packard, No. 5080-8730, King of Prussia, PA). The vials were stored in a freezer until required for GC analysis. Completeness of esterification was established by TLC analysis of the reaction mixture.

Equipment

GC analyses were carried out on a Hewlett-Packard 5880A level 4 flame ionization capillary GC, equipped with magnetic tape storage capability and a Model 7672A automatic sampler. The column used for all analyses was a Quadrex 100 m 0.25 mm I.D. SP 2340 glass column (Quadrex, New Haven, CT). (Analyses may now be accomplished alternatively on either of two recently introduced flexible columns: a flexible glass column with the same stationary phase (50 m Quadrex Monarch series) or a fused silica column with a similar but bonded phase (50 m Quadrex 007-CPS 1). Both alternatives allow efficient separation of FAME's in substantially reduced analysis time, and are more easily installed than the fragile soft glass column used in the present work.) Carrier gas was helium at a flow of 1 ml/min and make-up gas was nitrogen at a flow of 30 ml/min. The temperature program employed was 150 C-170 C at 0.4°/min, then 1°/min to 200 C, at which temperature the oven was held for a maximum of 40 min until all FAMEs had been eluted.

Determination of FAME Identity and Reference Standards

Initial identification of FAMEs was made by injecting samples from bovine tissue into an HP 5840 GC (located in the laboratory of H. Slover, USDA, ARS, BARC, Beltsville, MD) that had been calibrated by GC/mass spectroscopy to identify major components in the mixture (3). Verification of other constituents was made by peak enhancement of the unknown using authentic compounds and by retention time analysis of the unsaturated FAMEs before and after hydrogenation.

A reference standard with 18 known FAMEs common to bovine muscle (Nu-Chek Prep, Inc., Elysian, MN) contained the relative amount of each component approximately found in a polar fraction of a typical muscle sample. This standard was chromatographed after each group of 10 bovine samples to ascertain if changes in retention times and peak shape, due to instrumental changes during the analysis of the preceding block of samples, had occurred.

RESULTS AND DISCUSSION

The methodology described in this study was developed to undertake a project in lipid analysis involving over 200 bovine tissue samples taken from 2 feeding studies at Oklahoma State University (1). Our independent investigations involved detailed analyses of the fatty acids of the tissue samples generated in these 2 studies. In all, 49 animals were used in the 2 studies. In the dietary study, samples were obtained from 5 tissue loca-

tions in each grain-fed animal and from 2 corresponding tissue locations in each forage-fed animal (5). In the monensin study, samples were taken from 2 locations per animal. The systematic protocol developed for examining the bovine tissues from the dietary study is shown in Figure 1. A similar protocol was used for the monensin study. To indicate the magnitude of both studies, the total numbers of tissue samples, lipid fractions, and GC reports are shown in Figure 1. This figure shows the sequence used to handle the samples employed in this study up to the generation of the reports of the GC analyses. The continuation of this diagram, which describes the data manipulation to the generation of the final report, can be found in Figure 2 of the following article (6). Although samples of muscle and adipose tissue from 5 locations were studied, only tissue from ST and PM are represented on the flow chart for simplification. The same sequence was used to examine all other tissue samples. Each tissue was ground to a uniform consistency (use of a food processor minimized sampling problems) in the presence of antioxidant, packaged as accurately weighed replicate portions, and quickly frozen until required. All samples studied were extracted in duplicate (total 256 samples) (Fig. 1) for lipid by the sequential dry column method (2). Since this method allows for the recovery of separate neutral and polar lipid fractions, no further separations of the lipid extracts were required prior to derivatization. Excellent agreement between duplicates was obtained for amounts of recovered lipid and for total phosphorus in these samples (5).

In most previous studies, FAME profiles of bovine muscle have been reported as those obtained from total lipid extracts. Such extracts generally contain only small amounts of polar lipid, and consequently their contribution is diluted in the GC trace by the overwhelming presence of the neutral lipids. Additionally, polar lipid of bovine muscle contains large amounts of plasmalogen (up to 30%), which produce dimethylacetals (DMA) of long chain aldehydes by acid-catalyzed esterification techniques. Although the DMAs would appear as only trace artifacts in the GC trace of the FAMEs of total lipid extracts, they would appear as major components in a GC trace of polar lipid FAMEs. Therefore, to prevent the acid-catalyzed lysis of plasmalogen lipids in the polar lipid extract, a published method for transesterification of total lipids (4) was modified to handle phospholipid concentrates. Room temperature alkaline transesterification converts all phospholipids to esters while generating no DMAs from the plasmalogens. The neutral lipid fractions, which contain no plasmalogens, did not present such problems, and were derivatized to their FAMEs by the technique of Slover and Lanza (3).

The 256 tissue samples (Fig. 1) after sequential extraction yielded only 454 neutral and polar lipid fractions, not the expected 512, because certain adipose samples were extracted for total lipid. These fractions were, in turn, derivatized to FAMEs by the methods described above. Each FAME sample contained a measured amount of internal standard according to the method of Slover and Lanza (3).

GC Analysis of FAMEs

Prior to analysis of the FAME samples on the HP 5880A GC, a sample run program was devised to run the instrument unattended during the actual analysis and to store the data when each run was completed. A profile of that run program is shown in Figure 2. Although that program was developed for a specific instrument, it illustrates the steps that are needed to carry out unattended profiling on any automated GC. This run program was written (in Hewlett Packard BASIC) for neutral lipid analysis, but the program for polar lipids was similar. The first command of the program (line 10) was to print "prgm for neutral lipid extracts." All commands in the program written within quotation marks are printed on the chromatogram and are informational only. List commands (lines 60, 360, and 400) serve similar purposes and instruct the instrument to print such current information on the chromatogram. Lines 30-50 are another informational set which is programmed to print the checklist (lines 450-510) prior to the start of the unattended automated analysis. The lines 70-280 describe the program used to start and advance the automatic sampler. This program instructs the sampler to inject the reference mixture (line 210) after 4 samples have been run, and then once after every next 10 samples have been run. Lines 290-330 describe a sequence designed to reject a report and prevent its tape storage if the total area found is less than 10 area counts (line 330). The feature is designed to shut down the instrument if, for example, no injection occurred due to a broken syringe. If the report is satisfactory, it is transferred to tape (line 340) and the program advances to the next step (line 350), which is a loop wherein the unit prepares itself for the next injection.

The utility of obtaining fatty acid profiles of separate neutral and polar lipid fractions of the same tissue, as opposed to profiles of a *total* lipid extract, is demonstrated by the 3 chromatograms (Fig. 3) that were generated from the above run program. Peaks are identified by manually inserted peak numbers (Chromatogram B, Fig. 3) to overcome run-to-run variations in retention times and to facilitate subsequent handling of data. Chromatogram C is derived from a total lipid extract and is representative of what would be seen from a traditional Folch extraction (7). Chromatograms

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PROGRAM:     (ANNOTATION OFF)
10   PRINT " PRGM 5: PRGM FOR POLAR LIPID EXTRACTS."
15   REM  SAME AS PRGM 4, BUT WITHOUT STEPS 200,270.
20   FOR A=1 TO 11
30   READ P$
40   PRINT P$
50   NEXT A
60   LIST OVEN TEMP
70   INPUT "ENTER THE FIRST (N) AND LAST (M) BOTTLE # (N,M)",N,M
80   INPUT "ENTER THE DESIRED STOP #(1,2,OR 3) ",C
90   LET K=0
100  FOR I=N TO M STEP 2
110  LET K=K+1
120  IF K=5 THEN 150
130  IF K=10 THEN 430
140  GOTO 220
150  EDIT AUTO SEQ 4,1
160  EDIT AUTO SEQ 8,98
170  PRINT "-------------------------------------------------------------
180  PRINT "STOP# 1"
190  PRINT "REFERENCE VIAL #97"
210  START AUTO SEQ 97,97
220  EXECUTE X,"EDIT AUTO SEQ 4,"&VAL$(C)
230  EXECUTE X," EDIT AUTO SEQ 8, "&VAL$(I+1)
240  PRINT "-------------------------------------------------------------
250  PRINT "STOP# "&VAL$(C)
260  PRINT "SAMPLE VIAL #"&VAL$(I)
280  START AUTO SEQ I,I
290  LET S=0
300  FOR J=1 TO #PEAKS
310  LET S=S+AREA(J)
320  NEXT J
330  IF S<10 THEN 390
340  EXECUTE Y," SAVE REPORT "&VAL$(I)&" DEVICE# 16"
350  NEXT I
360  LIST CLOCK TIME
370  OVEN TEMP 70
380  STOP
390  PRINT "PROGRAM STOPPED BECAUSE NO PEAKS WERE FOUND IN LAST RUN"
400  LIST CLOCK TIME
410  OVEN TEMP 70
420  STOP
430  LET K=0
440  GOTO 220
450  DATA "() IS NEW TAPE CONDITIONED (DELETE DEVICE# 16)?"
460  DATA "() ARE GASES OK?","() IS PAPER SUPPLY OK?"
470  DATA "() ARE SAMPLES LOADED AND STIRRED?","() IS SAMPLE TBL OK?"
480  DATA "() ARE SOLVENT VIALS FULL?","() IS REFERENCE VIAL FULL?"
490  DATA "() ARE OTHER VIALS RETURNED TO FREEZER?","() IS SEPTUM OK?"
500  DATA "() IS THE FID IGNITOR CONTROL OFF?"
510  DATA "() IS THE OVEN TEMP NOW AT THE PROPER INITIAL VALUE?"
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FIG. 2. Sample program for automated gas chromotograph.

A and B are derived from the neutral and polar lipid fractions, respectively, of the sequential extraction (2) of the same tissue. The advantage of examining separate chromatograms (A and B) is illustrated by the difference in fatty acid constituents associated with each lipid fraction. Specifically, the polyunsaturated fatty acids (peaks 380, 420, 460-660) are concentrated in the polar lipid fraction (B), but are overwhelmed by the contribution of the fatty acids of the neutral fraction when examined as a part of a total lipid extract (C). To achieve a fatty acid profile of the total lipid—with the content of the polyunsaturated acids obtained in detail—the data of the separate neutral and

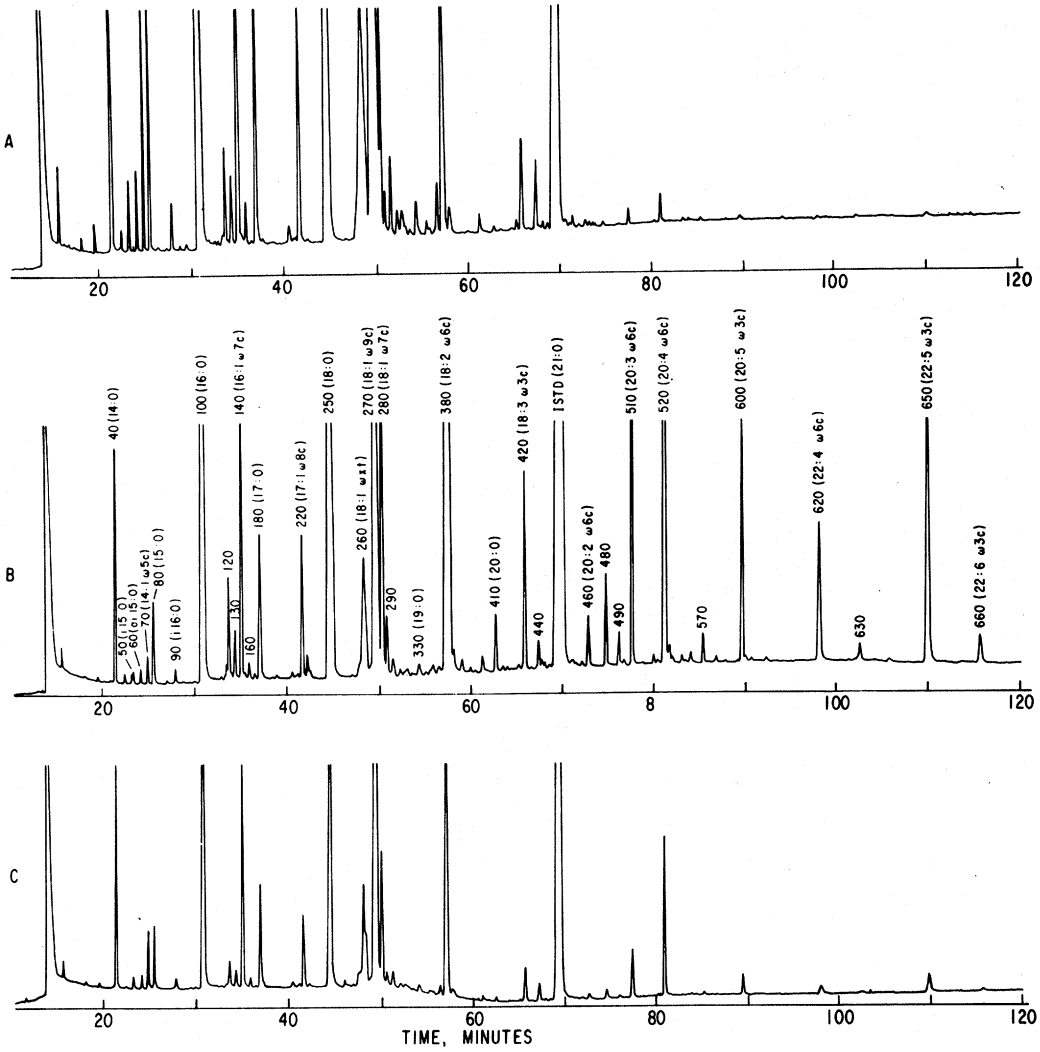

FIG. 3. Comparison of capillary GC chromatograms for neutral, polar, and total FAME extracts of bovine muscle tissue.
Trace A: Neutral FAMEs;
Trace B: Polar FAMEs (showing peak numbers and identities);
Trace C: Total FAMEs.

polar runs need only be consolidated.

At the end of each GC run, each trace was checked for completeness of integration of each component against the values in the sample report. Criteria used to judge the acceptability of each report have been described (3). Data for each run was, in turn, stored on magnetic tape. The stored data did not include the assigned peak numbers, which were entered manually as described in the next report (6).

We have demonstrated that fatty acid profiling on such a massive scale requires deliberate planning from tissue handling on through GC analysis. This profiling has been accomplished by use of a strict protocol for tissue comminution, by the dry-column method of lipid extraction and concomitant class separation, and by capillary column GC analysis that is automated for unattended operation and data storage.

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Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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